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### Remarks:

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(54) **Plasmids for the production of transgenic plants that are modified in habit and yield**

(57) Plasmids that comprise a DNA sequence coding for a protein that modifies the metabolism of soluble sugars, for example, an inorganic pyrophosphatase agent and plant cells comprising those plasmids, for the

production of transgenic plants that are modified in habit and yield are described, the plants being modified in habit by the transfer and the expression of genes that intervene in sugar metabolism or sugar distribution inside a plant.

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**Description**

[0001] The present invention relates to plasmids for the production of transgenic plants that are modified in habit and yield.

5 [0002] The growth, development and yield of a productive plant or an ornamental plant depend on the energy that the plant obtains by fixing CO<sub>2</sub> in carbohydrates during photosynthesis. The primary locations for photosynthesis are the leaf and, to a lesser extent, the stem tissue, whereas other plant organs, such as roots, seeds or tubers, make no substantial contribution to the formation of photoassimilates but, on the contrary, are dependent as regards their growth 10 on being supplied by photo-synthetically active organs. That means that there is a flow of photosynthetically obtained energy from photosynthetically active tissues to photosynthetically inactive parts of a plant.

10 [0003] The photosynthetically active tissues are referred to as sources. They are defined as net exporters of fixed carbon dioxide. The photosynthetically inactive parts of a plant are referred to as "sinks". They are defined as net importers of photosynthetically fixed carbon dioxide.

15 [0004] It is to be assumed that both the efficient use of products of photosynthesis and their distribution within a plant have a strong influence on the plant in several respects. The habit of plants may be mentioned as an example. Newly developing organs such as very young leaves, or other regions such as the root and seeds, are completely dependent on the photosynthetic capacity of the sources. That means that the development of such organs is dependent on the distribution inside the plant of the photoassimilates formed in the sources. The possibility of forming young leaves, or 20 of forming roots, could have drastic effects on the habit of a plant, such as, for example, the size of a plant, the internodal distance, the size and shape of a leaf, the appearance of a leaf and the amount and shape of the root formed. Furthermore, the distribution of photoassimilates is assumed to be of very crucial importance for the yield of a plant. Thus, 2 the total photosynthetic capacity of wheat has not changed substantially in the last few decades, whereas the harvestable yield of wheat plants for humans has increased. This is largely attributable to the fact that the ratio between competing sinks has been changed to the effect that the sinks that are important for the yield, such as seeds, take up 25 considerably more photoassimilate than do other regions of the plant that are not important for the yield, such as, for example, the stalk. It was thus possible by, in this case, shortening the haulm to obtain in wheat a sink to source ratio that is much more favourable for humans. That underlines the importance in higher plants, in relation to both the habit and the yield of plants, of the distribution of photoassimilates formed in the primary sources.

30 [0005] Modifications in habit, resistance to dryness and/or frost and especially modification in the yield of plants are considerable improvements over known plants.

[0006] Biotechnological processes for the genetic modification of dicotyledonous and monocotyledonous plants are known (Gasser and Fraley, 1989, *Science* 244, 1293-1299).

35 [0007] It is not known what biochemical mechanisms regulate the ratio of sink to source.

[0008] An object of the present invention is to provide plants that are modified in their habit, such as size, leaf shape, internodal distance, cell wall structure, seed, bulb and root formation, and especially in their yield on harvesting.

[0009] The present invention provides a plant which comprises a DNA coding for a protein that modifies sugar metabolism or sugar distribution. The DNA sequence is expressiv in the resulting transgenic plant, which leads, for example, to an increase, in those transgenic plants, in the proportion of photoassimilates that are present in the form of soluble sugars in the source leaves.

40 [0010] For the purpose, plasmids are provided that comprise a DNA sequence coding for a protein that modifies the metabolism of soluble sugars, those plasmids being inserted into plant cells and those cells being regenerated to whole plants. The product coded for by the DNA sequence intervenes in the phosphate/pyrophosphate metabolism, the DNA sequence being a DNA sequence of an inorganic pyrophosphatase gene.

[0011] In most plants, photoassimilates are distributed within a plant in the form of sugars, and preferentially in the 45 form of sucrose. Because sucrose is the most important form of transport for carbohydrates from source to sinks, another important determinant for the strength of a source, and therefore the efficient supply to sinks, could be the availability and content of soluble sugars (various mono-, di- and tri-saccharides, such as, for example, fructose, glucose and sucrose) in the leaves. For example, a relatively high content of sucrose in the source leaves should result in an increased supply to sinks and, accordingly, also in an increased proportion of photoassimilates in the harvestable organs. That would lead to an increase in yield.

50 [0012] The ratio between soluble sugars and starch in the source leaves is assumed to be of decisive importance for the distribution of carbohydrates between sink and source parts of a plant. An efficient supply of sinks with sucrose from the source leaves should lead to a modification in the habit of the plant, but especially to an increase in yield, which is in most cases determined by the sink store, such as seed and tuber or root.

55 [0013] One of the important control points in sucrose biosynthesis is the conversion of fructose-1,6-diphosphate (Fru-1,6-P2) into fructose-6-phosphate (Fru-6-P). One of the enzymes involved in that step is the pyrophosphate:fructose-6-phosphate-1-phototransferase (PFP). That enzyme can on the one hand convert Fru-1,6-P2 into Fru-6-P, with the release of inorganic pyrophosphate, and can on the other hand phosphorylate Fru-6-P into Fru-1,6-P2,

with the consumption of inorganic pyrophosphate. The direction of the reaction, which is catalysed by PFP, is controlled by the relative content of inorganic pyrophosphate and inorganic phosphate.

5 [0014] Continuous removal of inorganic pyrophosphate by an inorganic pyrophosphatase should bring about a shift in the above equilibrium in the direction of Fru-6-P and accordingly an increased formation of soluble sugars, such as, for example, hexoses and/or sucrose. A shift in the distribution of the photoassimilates by removing the pyrophosphate using an inorganic pyrophosphatase should also cause in reaction, catalysed by the UDP-glucose-pyrophosphorylase,



10 to be shifted in the direction of UDP glucose. The thus increased provision of UDP glucose leads to other modified properties of the plant, such as, for example, thicker cell walls. That is effected by increased provision of the precursors for cell wall biosynthesis. The thickening of the cell walls leads to an increase in the resistance to dryness in those plants, which is brought about by a reduced rate of transpiration.

15 [0015] A large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR 322, pUC series, M13 mp series, pACYC 184, etc.. Accordingly, the sequence can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence 20 analysis, restriction analysis, electrophoreses and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right borders but often the right and the left border of 25 the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted.

[0016] The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema, In: The Binary Plant Vector System Offset-drukkerij Kinters B.V., Alblasterdam, 1985, Chapter V; Fraley *et al.*, Crit. Rev. Plant Sci., 4: 1-46 and An *et al.*, EMBO J. (1985) 4: 277-287.

30 [0017] Once the inserted DNA has been integrated in the genome it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418, bleomycin, hygromycin or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

35 [0018] It has now been found that a plasmid that comprises a DNA sequence that can be fused to the regulatory region of one or more other genes capable of bringing about expression of the gene in a plant cell or in a plant. The regulatory region comprises the promotor region of a plant gene and the termination signal of the same or of a different plant gene.

40 [0019] It is possible to use a promotor, for example, the cauliflower mosaic virus promotor (CaMV) that brings about constitutive expression and a plant termination signal. Other possible promtors are promotors encoding an expression specifically only in photosynthetically active cells (e.g. the ST-Ls1 promotor, Stockhaus *et al.*, EMBO J. 8, 2445-2451) which should be a special advantage when a change in the sucrose metabolism is to be reached in leaves, a source-specific promotor active only during loading of sink-organs (i.e. at a special developmental phase) a root-specific promotor; if a specific expression in roots is advantageous due to e.g. a thicker cell wall necessary, a storage-sink-specific promotor (being active e.g. only in tubers of potato, tap root of sugar beet, fruits of tomato like is the case for the class 45 I patatin promotor) if the changes to be achieved are specifically advantageous for a sink tissue.

[0020] A plant termination signal may comprise the 3'-end of the poly-A side of the octopine synthase gene.

50 [0021] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection or electroporation as well as other possible methods. If agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors 55 can replicate themselves both in *E. coli* and in agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into agrobacteria (Holsters *et al.*, Mol. Gen. Genet. (1978), 163: 181-187). The agrobacterium used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-

DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with Agrobacterium tumefaciens or Agrobacterium rhizogenes for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain 5 antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[0022] The transformed cells grow inside the plants in the usual manner. The plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The 10 resulting hybrid individuals have the corresponding phenotypic properties.

#### Terms and abbreviations

#### Abbreviations

##### [0023]

|          |   |
|----------|---|
| bp, kb = | base pairs, kilobases                                 |
| DNA =    | deoxyribonucleic acid, carrier of genetic information |
| HEPES =  | N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid  |
| SDS =    | sodium dodecyl sulphate                               |
| tris =   | tris(2-aminoethyl)amine                               |
| EDTA =   | ethylenediaminetetraacetic acid                       |
| U =      | unit (enzyme unit)                                    |

[0024] The following plasmids were deposited at the Deutsche Sammlung von Mikroorganismen (DSM) in Braunschweig, Federal Republic of Germany on 20.08.1990 and on 10.10.1991 (deposit number

plasmid p35S-Ω-ppase (DSM 6141) - 20.08.1990

30 plasmid L 700 : ppa (DSM 6733) - 10.10.1991

#### Description of the Figures

##### [0025] Figure 1

35 shows the structure of the 4.0 kb plasmid p35S-Ω-ppase. The plasmid comprises the following fragments:

A = Fragment A (529 bp): contains the 35S promoter of cauliflower mosaic virus (CaMV). That fragment includes the nucleotides 6909 to 7437 of CaMV.

40 B = Fragment B (73 bp) : contains 61 nucleotides of sequence (Seq. ID No. 1):

TTTACAACATTACCAACAACAAACAACAAACATTACATTACTATTACAATTAA the Asp 718 linker of sequence GGTACC is located at the 5'-end of the above sequence and the Ncol linker of sequence CCATGG is located at the 3'-end.

45 C = Fragment C (554 bp): contains the protein-coding region of the inorganic pyrophosphatase (ppa) from E. coli (nucleotides positions +39 to +565 of the sequence according to Lahti et al.).

D = Fragment D (192 bp): contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5.

50 [0026] The cleavage sites described in the Example are also shown.

##### [0027] Figure 2

shows the detection of the RNA, coded for by the pyrophosphatase gene, in transgenic potato and tobacco plants by means of Northern blot analysis.

55 Positions 1 to 19 : Samples of the total RNA obtained from leaves of potato plants that have been transformed with plasmid p35S-Ω-ppase and regenerated

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positions 20 to 28: Samples of the total RNA obtained from leaves of tobacco plants that have been transformed by the plasmid p35S-Ω-ppase and regenerated

5 Position A : Sample from an untransformed potato plant

Position B : Sample from an untransformed tobacco plant

[0028] The black spots indicate the RNA coded for by the pyrophosphatase.

[0029] Figure 3

10 shows the detection of the occurrence of new pyrophosphatase activity in protein extracts from leaves of the transgenic potato and tobacco plants transformed by the p35S-Ω-ppase plasmid, in a SDS polyacrylamide gel.

Positions 1 to 7 : Protein extract from leaves of transformed potato plants

15 Positions 8 and 9 : Protein extract from leaves of transformed tobacco plants

Position A : Protein extract from leaves of untransformed tobacco plants

20 Positions B and C : Protein extract from leaves of untransformed potato plants

Positions X and Y : Protein extract from E. coli

[0030] Figure 4

25 shows a comparison between the content of glucose, fructose, sucrose and starch in mmol/m<sup>2</sup> in a tobacco plant (  ) that expresses the 35S-Ω-ppase gene and in an untransformed tobacco plant (  ) in leaves of different age.

[0031] In the Figure:

1 = very young leaves

2-4 = mature leaves

30 5-6 = old leaves

[0032] Figure 5

shows the effect of the expression of the 35S-Ω-ppase gene in transgenic potato plants (positions 1 to 12) on the sucrose/starch ratio in the leaves of transformed plants.

35 C = untransformed potato plant

Figure 6

[0033] Shows the structure of the 5.0 kb plasmid L 700 : ppa.

40 [0034] The plasmid comprises the following fragments:

A= Fragment A (1585 bp): contains the promotor of the ST-LS1 gene. That fragment includes the nucleotides, positions +1 to +1585 of the ST-LS1 gene.

45 B= Fragment B (528 bp): contains the protein-coding region of the inorganic pyrophosphatase (ppa). The fragment comprises the nucleotides, positions +39 to +565.

C= Fragment C (192 bp): contains the polyadenylation signal of gene 3 of the T-DNA of the Ti plasmid pTiACH5, nucleotides, positions 11749- 11939.

50 [0035] The cleavage sites described in the Examples are also shown.

[0036] In order better to understand the Examples forming the basis of this invention, all the processes that are necessary for these tests and which are known per se will first of all be listed:

55 1. Cloning process

The vector pUC18 (Yanisch-Perron et al., Gene (1985), 33, 103-119) was used for cloning.

For plant transformation, the gene constructions were cloned into the binary vector BIN19 (Bevan, Nucl. Acids Res. (1984), 12, 8711-8720).

## 2. Bacterial strains

The *E. coli* strains BMH71-18 (Messing *et al.* Proc. Natl. Acad. Sci. USA (1977), 24, 6342-6346) or TB1 were used for the pUC vectors. TB1 is a recombination-negative, tetracycline-resistant derivative of strain JM101 (Yanisch-Perron *et al.*, Gene (1985), 33, 103-119). The genotype of the TB1 strain is (Bart Barrel, pers. communication): F (traD36, proAB, lacI, lacZΔM15), Δ(lac, pro), SupE, thi, recA, Sri::Tn10(Tc<sup>R</sup>).

The plant transformation was carried out by means of the *Agrobacterium tumefaciens* strain LBA4404 (Bevan, M., Nucl. Acids Res. 12, 8711-3721, (1984); BIN19 derivative).

3. Transformation of *Agrobacterium tumefaciens*.

In the case of BIN19 derivatives, the insertion of the DNA into the agrobacteria was effected by direct transformation in accordance with the method developed by Holsters *et al.*, (Mol. Gen. Genet. (1978), 163, 181-137). The plasmid DNA of transformed agrobacteria was isolated in accordance with the method developed by Birnboim and Doly (Nucl. Acids Res. (1979), 7, 1513-1523) and was separated by gel electrophoresis after suitable restriction cleavage.

## 4. Plant transformation

**A) Tobacco:** 10 ml of an overnight culture of *Agrobacterium tumefaciens*, grown under selection, were centrifuged off, the supernatant was discarded, and the bacteria were resuspended in the same volume of antibiotic-free medium. In a sterile Petri dish, leaf discs of sterile plants (approximately 1 cm<sup>2</sup>), the central vein of which had been removed, were immersed in that bacterial suspension. The leaf discs were then placed in a closely packed arrangement in Petri dishes containing MS medium (according to Murashige and Skoog, Physiologia Plantarum (1962), 15, 473-497) with 2 % sucrose and 0.8 % Bacto agar. After two days' incubation in the dark at 25°C, they were transferred onto MS medium containing 100 mg/l of kanamycin, 500 mg/l of cloran, 1 mg/l of benzylaminopurine (BAP), 0.2 mg/l of naphthylacetic acid (NAA) and 0.8 % Bacto agar. Growing shoots were transferred onto hormone-free MS medium with 250 mg/l of cloran.

**B) Potato:** 10 small leaves, damaged with a scalpel, of a sterile potato culture were placed in 10 ml of MS medium with 2 % sucrose containing from 30 to 50 µl of an *Agrobacterium tumefaciens* overnight culture grown under selection. After from 3 to 5 minutes' gentle shaking, the Petri dishes were incubated in the dark at 25°C. After 2 days, the leaves were laid out on MS medium with 1.6 % glucose, 2 mg/l of zeatin ribose, 0.02 mg/l of naphthylacetic acid, 0.02 mg/l of gibberellic acid, 500 mg/l of cloran, 50 mg/l of kanamycin and 0.8 % Bacto agar. After incubation for one week at 25°C and 3000 lux, the cloran concentration in the medium was reduced by half. Further cultivation was effected in the manner described by Rocha-Sosa *et al.* in EMBO Journal 8, 29 (1989).

## 5. Analysis of genomic DNA from transgenic plants

The isolation of genomic plant DNA was effected in accordance with Rogers and Bendich (Plant Mol. Biol. (1985), 5, 69-76).

For the DNA analysis, after suitable restriction cleavage, from 10 to 20 µg of DNA were analysed by means of Southern blotting for the integration of the DNA sequences to be investigated.

## 6. Analysis of the total RNA from transgenic plants

The isolation of plant total RNA was carried out in accordance with Logemann *et al.* (Analytical Biochem. (1987), 163, 16-20).

For the analysis, 50 µg portions of total RNA were investigated by means of Northern blotting for the presence of the transcripts sought.

## 7. Protein extraction

For the extraction of total protein from plant tissue, pieces of tissue were homogenised in protein extraction buffer (25 mM sodium phosphate pH 7.0, 2 mM sodium hydrogen sulphite, 2 mM phenylmethylsulphonyl fluoride (PMSF)), with the addition of 0.1 % (w/v) of insoluble polyvinylpyrrolidone (PVP).

After filtering through cellulose, cell detritus was centrifuged off for 20 minutes at 10,000 revolutions per minute and the protein concentration of the supernatant was determined in accordance with the method developed by Bradford (Anal. Biochem. (1976) 72, 248-254).

8. Detection of the inorganic pyrophosphatase activity (modified according to Baykov *et al.*, Analytical Biochemistry 171, 271-276 (1988))

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The total protein was extracted from plants as described under point 7, and unmodified SB buffer (125 mM tris/HCl pH 6.8, 10 % 2-mercaptoethanol, 20 % glycol, 0.004 bromophenol blue) was added and the whole was added to 10 % SDS polyacrylamide gels. The mixtures were denatured, not by heating, before separation in the SDS polyacrylamide gels. After electrophoretic separation, the gels were rinsed briefly in water and incubated for 1 hour at 37°C in pyrophosphate buffer (0.05 M tris/HCl pH 9.0; 0.03 mM inorganic pyrophosphate ( $\text{Na}_4\text{P}_2\text{O}_7$ ), 5 mM  $\text{MgCl}_2$ ). 17 % by volume staining powder (140 mg of ammonium molybdate, 11.5 mg of malachite green in 10 ml of 2.5 M  $\text{H}_2\text{SO}_4$ ) was then added to the solution. The formation of a turquoise precipitate indicated pyrophosphatase activity.

10 9. Determination of sucrose, glucose, fructose and starch

a) Extraction

15 [0037] Small leaf discs (diameter 10 mm) frozen in liquid nitrogen were extracted for 30 minutes at 80°C in 0.5 ml of buffer (80 % (v/v) ethanol; 10 mM HEPES pH 7.5) in a water bath. The supernatant containing the soluble components was poured off and the volume was determined. The supernatant was used to determine the soluble sugars.

[0038] The insoluble material that remained was rinsed with water and finely ground in a mortar. The extract was then boiled for 1 hour at 95°C in 0.2 M potassium hydroxide solution, neutralised with 70  $\mu\text{l}$  of 1N acetic acid and then centrifuged. Aliquots of the resulting starch solution were used to determine the starch.

20 b) Quantitative determination of soluble glucose, fructose and sucrose

[0039] The quantitative determination of soluble glucose, fructose and sucrose was carried out in the following test mixture:

|                       |  |
|-----------------------|--|
| 100.0 mM              | imidazole-HCl, pH 6.9                        |
| 1.5 mM                | $\text{MgCl}_2$                              |
| 0.5 mM                | NADP <sup>+</sup>                            |
| 1.3 mM                | ATP  |
| 10-50.0 $\mu\text{l}$ | sample                                       |
| 1.0 U                 | glucose-6-phosphate-dehydrogenase from yeast |

35 [0040] The mixture was incubated for five minutes. The determination of the sugars was then carried out photometrically by the successive addition of

|        |   |
|--------|---|
| 1.0 U  | hexokinase from yeast (for the determination of glucose)                  |
| 1.0 U  | phosphoroglucose isomerase from yeast (for the determination of fructose) |
| 20.0 U | invertase from yeast (for the determination of sucrose).                  |

40 c) Starch determination

[0041] Hydrolytic enzymes were added at 55°C to the starch solution obtained after the ethanolic extraction under a) and the whole was incubated for twelve hours in the following mixture:

|         |  |
|---------|--|
| 50.0 mM | sodium acetate, pH 4.8                         |
| 1.4 U   | amyloglucosidase from <u>Aspergillus niger</u> |
| 2.0 U   | $\alpha$ -amylase from porcine pancreas        |

50 [0042] After incubation, the insoluble constituents were removed by 4 minutes' centrifugation at 16,000 g. In the supernatant, the resulting glucose was then determined enzymatically, as described under b).

Example 1

55 Preparation of plasmid p35S- $\Omega$ -ppase and insertion of the plasmid into the plant genome

[0043] A DNA sequence from E. coli K12 that codes for inorganic pyrophosphatase was provided with a promoter

of the 35S RNA of cauliflower mosaic virus (CaMV) that brings about constitutive expression, a DNA segment from tobacco mosaic virus that serves as a translation amplifier, and a plant termination signal. The plant termination signal comprises the 3'-end of the poly-A side of the octopine synthase gene. The vicinity of the translation initiation codon ATG of the sequence for inorganic pyrophosphatase was subjected to a controlled mutagenesis in order to achieve optimum expression in eukaryotic cells. Plasmid p35S- $\Omega$ -ppase comprises the four fragments A, B, C and D which were cloned into the cleavage sites of the polylinker of pUC 18 (Figure 1).

[0044] Fragment A (529 bp) contains the 35S promoter of cauliflower mosaic virus (CaMV). That fragment includes the nucleotides 6909 to 7437 of CaMV (Franck *et al.*, Cell 21, 285-294). It was isolated in the form of Eco RI-Kpn I fragment from plasmid pDH51 (Pietrzak *et al.*, Nucleic Acids Research 14, 5857-5868) and cloned between the Eco RI-Kpn I cleavage sites of the polylinker of plasmid pUC 18.

[0045] Fragment B contains a segment consisting of 61 nucleotides having the sequence (Seq. ID No.1):

TTTACAACAATTACCAACAACAACAAACAACAAACATTACAATTACTATTACAATTAA

which is homologous to a portion of the DNA segment, serving as a translation amplifier, from the tobacco mosaic virus strain U (Gallie *et al.*, Nucleic Acids Res. 15, 3257-3273). That segment, which was produced by DNA synthesis, was provided at the 5'-end with an Asp 718 linker having the sequence

GGTTACCG

and at the 3'-end with an Nco I-linker having the sequence

CCATGG.

[0046] Fragment B was cloned between the Kpn I and Sma I cleavage site of the polylinker of pUC 18.

[0047] Fragment C includes the protein-coding region of inorganic pyrophosphatase (ppa) from *E. coli* which comprises the nucleotides, positions -39 to +565, of the sequence according to Lahti *et al.*, (J. Bacteriology 170, 5901-5907).

[0048] Fragment C was cloned between the Nco I site of fragment B and the Sal I site of the polylinker of pUC 18.

[0049] Fragment D (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti plasmid pTiACH5 (Gielen *et al.*, EMBO J. 3, 835-846), nucleotides 11749-11939, which was isolated in the form of Pvu II-Hind III fragment from plasmid pAGV 40 (Herrera-Estralla *et al.*, (1983) Nature 303, 209-213) and, after the addition of Sph I linkers to the Pvu II cleavage site, was cloned between the Sph I-Hind III cleavage sites of the polylinker of pUC 18.

[0050] The size of plasmid p35S- $\Omega$ -ppase is 4.0 kb.

[0051] Plasmid p35S- $\Omega$ -ppase was inserted into binary vectors and then inserted into tobacco and potato plants by means of the agrobacteria system. Intact and fertile plants were regenerated from transformed cells.

[0052] Analysis of the transformed plants by Southern blot analysis indicated the presence of the intact chimaeric pyrophosphatase gene in the transgenic plants.

[0053] In order to detect the RNA coded for by the pyrophosphatase gene in transgenic potato and tobacco plants, 30  $\mu$ g of total RNA were isolated from the leaves of the transformed and regenerated potato and tobacco plants and from untransformed potato and tobacco plants. The RNA was investigated by means of Northern blot analysis. The analysis of the regenerated plants (potato: positions 1, 2, 8, 11, 12, 15 and 13; and tobacco: positions 21 to 26) indicated, in the tissues, RNA that hybridises specifically with the coding sequence of inorganic pyrophosphatase and that is absent in untransformed plants (potato plant, position A, tobacco plant, position B) (Figure 2).

[0054] The detection of the new pyrophosphatase activity in protein extracts from leaves of the transgenic potato and tobacco plants transformed with the p35S- $\Omega$ -ppase plasmid was carried out in partially denatured gels, as described under point 8.

[0055] The protein extracts of transformed plants that comprise an RNA hybridising specifically with the coding sequence of inorganic pyrophosphatase exhibited an inorganic pyrophosphatase activity (potato plants: positions 1 to 7 and tobacco plants: positions 8 and 9) which was not present in untransformed plants, positions A to C (see Figure 3)

[0056] Transgenic tobacco and potato plants were thus produced that comprise a new inorganic pyrophosphatase activity that originates from the *E. coli* gene inserted into those plants (see Figure 3).

[0057] The regenerated tobacco and potato plants exhibited a number of differences in respect of phenotypic and biochemical parameters.

## a) Transgenic tobacco plants

Transgenic tobacco plants having a high level of expression of the pyrophosphatase gene exhibited a marked reduction in plant size, while tobacco plants having a medium level of expression of the pyrophosphatase gene exhibited only a slight reduction in size. The compactness is not attributable to a reduction in the number of leaves but to a reduction in the internodal distance.

The young leaves of the tobacco plants that express pyrophosphatase did not exhibit any marked phenotypic differences in comparison with untransformed control plants. The older leaves of the pyrophosphatase-expressing leaves, on the other hand, exhibited a marked thickening of the leaf. In plants that express the pyrophosphatase gene to a very high degree, a bleaching of the older leaves was observed. Apart from those phenotypic differences, the tobacco plants that express the pyrophosphatase gene exhibited a marked change in the composition of the carbohydrates in leaves of different age (see Figure 4). For example, the amounts of soluble sugars, especially glucose, fructose and sucrose, were distinctly increased in all leaves compared with leaves of untransformed plants, an increase by a factor of up to 20-50 being observed in the older leaves. At the same time, at least in the older leaves, there was an increase in the amount of starch formed, but that increase was not as great as the increase in the proportion of soluble sugars, so that, overall, on the one hand a marked increase was observed in the total content of starch and soluble sugars as a result of the expression of pyrophosphatase and, on the other hand, there was a clear shift in the distribution of the photoassimilates between starch and soluble sugars towards soluble sugars.

## b) Transgenic potato plants

Transgenic potato plants expressing the pyrophosphatase gene exhibited as the first substantial phenotypic modification a markedly compact size. The compactness is accompanied by increased branching of the plants owing to the increased formation of axial shoots. It is obvious that this compact growth has many advantages with regard to stability and sensitivity to wind.

The potato plants that express the pyrophosphatase gene also exhibited drastic changes in respect of the composition of the carbohydrates in the leaf (see Figure 5). For example, the potato plants have an increased sucrose:starch ratio. The increase in that ratio can be as much as a factor of 20. Unlike the tobacco plants, the transgenic potato plants did not exhibit a drastic increase in hexoses (glucose and fructose).

As a result of the expression of the gene coding for inorganic pyrophosphatase in transgenic potato plants, it was possible to achieve a modification in the sucrose/starch ratio and accordingly to modify the capacity of the source leaf.

**[0058]** The pyrophosphatase gene can be cloned from many other sources and be used for similar experiments. In addition the primary sequence of the pyrophosphatase can be modified to achieve a higher expression level and/or to target the ppase into e.g. other subcellular organelles (chloroplasts, mitochondria, vacuole, extracellular space) or to use other promoters ensuring the expression specifically only in e.g. photosynthetically active cells, in seeds, tubers, tap roots, fruits, roots, stem, flowers etc. or under specific environmental conditions such as drought, heat, cold, high-salt soils etc.

**Example 2**

## Preparation of plasmid L700:ppa and insertion of the plasmid into the plant genome

**[0059]** A DNA sequence from *E. coli* K12 that codes for inorganic pyrophosphatase was provided with a promoter of the ST-LS1 gene (Stockhaus et al., EMBO J. 8, 2445-2451 (1989)) that brings about specific expression in photosynthetically active cells, and a plant termination signal. The plant termination signal comprises the 3'-end of the poly-A side of the octopine synthase gene. Plasmid L700:ppa comprises the three fragments A, B and C which were cloned into the cleavage sites of the polylinker of pUC 18 (Figure 6).

**[0060]** Fragment A (1585 bp) contains the promoter of the ST-LS1 gene (op.cit.). That fragment includes the nucleotides +1 to +1585 of the ST-LS1 gene (Eckes et al., Mol.Gen.Genetics 205, 14-22). It was isolated in the form of a EcoRI-MboI fragment and cloned between the EcoRI-SmaI cleavage sites of the polylinker of plasmid pUC 18 after the MboI site had been flushed by T4-DNA Polymerase.

**[0061]** Fragment B includes the protein-coding region of inorganic pyrophosphatase (ppa) from *E. coli* which comprises the nucleotides, positions +39 to +565, of the sequence according to Lahti et al., (J. Bacteriology 170, 5901-5907). It was isolated in the form of a NcoI - SalI fragment from the plasmid p35S-ppase (cf. fig. 1). Fragment B was cloned between the BamHI site and the SAlI site of the polylinker of pUC 18 after the NcoI site and the BamHI site had been made blunt end by fill-in reactions.

**[0062]** Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti plasmid pTiACH5

(Gielen et al., EMBO J. 3, 835-846) nucleotides 11749-11939, which was isolated in the form of a Pvull-HindIII fragment from plasmid pAGV 40 (Herrera-Estrella et al., (1983) Nature 303, 209-213) and, after the addition of SphI linkers to the Pvull cleavage site, was cloned between the SphI-HindIII cleavage sites of the polylinker of pUC 18.

[0063] The size of plasmid L700:ppa is 5.0 kb.

5 [0064] Plasmid L700:ppa was inserted into binary vectors and then inserted into tobacco and potato plants by means of the agrobacteria system. Intact and fertile plants were regenerated from transformed cells.  
[0065] Analysis of the transformed plants by Southern blot, RNA blot and zymogram analysis indicated the presence and expression of the pyrophosphatase gene in the transgenic plants only in leaf tissue respectively photosynthetically active cells.  
10 [0066] The specific expression of the *E. coli* pyrophosphatase in photosynthetically active cells (leaf) leads to an increased supply of sink organs (roots, tubers) with sucrose and thus increases yield.

**Claims**

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1. Use of a DNA molecule coding for an inorganic pyrophosphatase fused to the regulatory regions of one or more other gene(s) capable of bringing about the expression of said DNA sequence in plant cells or plants for the transformation of plant cells or the production of transgenic plants.

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2. The use of a DNA molecule according to claim 1, wherein said DNA molecule encodes an inorganic pyrophosphatase from *E. coli*.

3. The use of a DNA molecule according to claim 1 or 2, wherein the regulatory regions comprise promoter regions and transcriptional termination signals.

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4. The use of a DNA molecule according to claim 3, wherein the terminator signal comprises the 3'-end of the poly-A side of the octopine synthase gene.

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5. The use of a DNA molecule according to claims 3 or 4, wherein said promoter is selected from the group consisting of:

a promoter of a plant gene,  
a promoter of a viral gene,  
a promoter bringing about constitutive expression,  
35 a promoter bringing about expression in photosynthetically active cells,  
a promoter bringing about expression in cells of storage sinks,  
a promoter bringing about expression in cells of roots,  
the promoter of the 35S RNA of cauliflower mosaic virus,  
the ST-LS1 promoter, and  
40 the class I patatin gene promoters.

6. A method for the production of transgenic plants with a modified sugar metabolism comprising the following steps:

45 a) transforming plant cells with a DNA molecule as defined in any of claims 1 to 5, and  
b) using said transformed plant cell to regenerate transgenic plants.

7. The method according to claim 6, wherein said plant cell is a potato cell.

8. The method according to claim 6, wherein said plant cell is a tobacco cell.

50 9. A transgenic plant cell comprising a DNA sequence as defined in any of claims 1 to 5.

10. The transgenic plant cell according to claim 9, which is a potato cell.

55 11. The transgenic plant cell according to claim 9, which is a tobacco cell.

12. The use of the transgenic plant cell according to any of claims 9 to 11 for the regeneration of a transgenic plant.

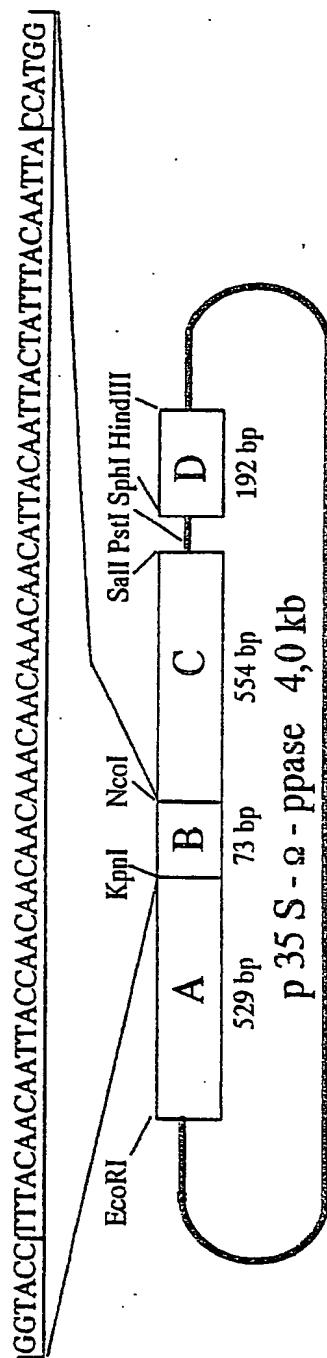


Fig. 1

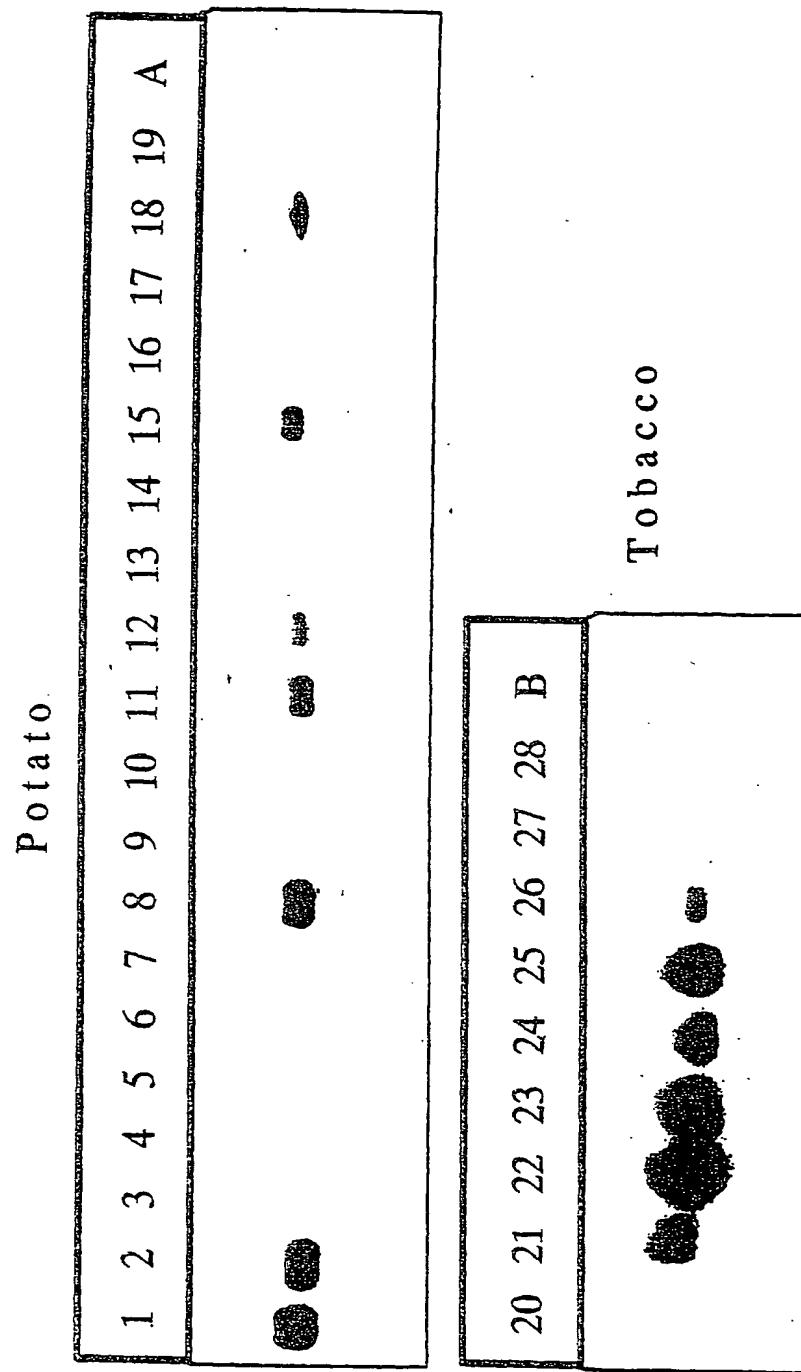


Fig. 2

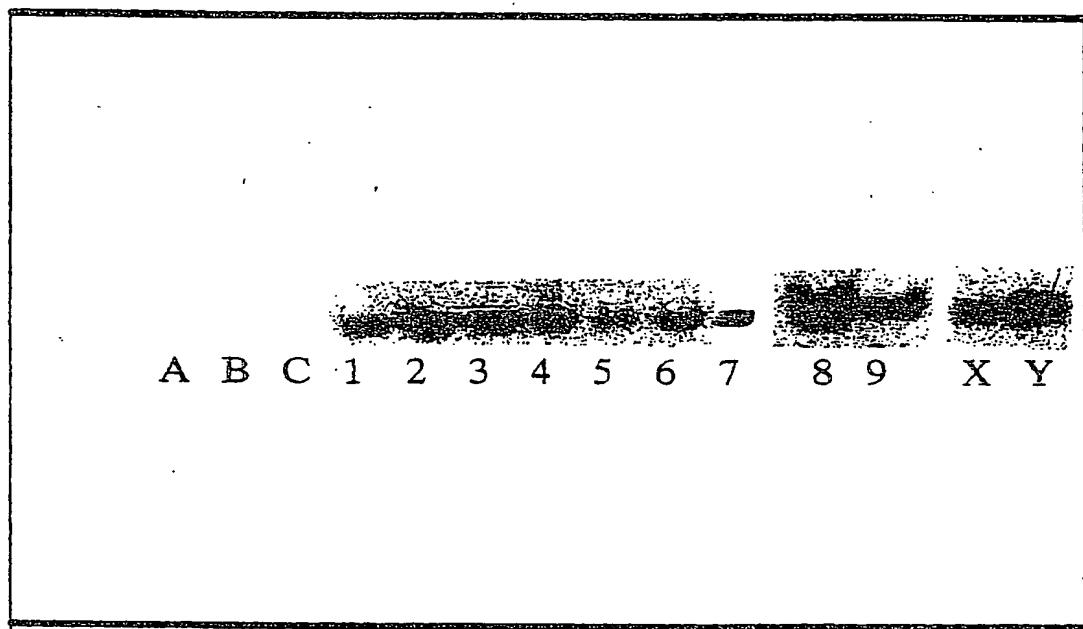


Fig. 3

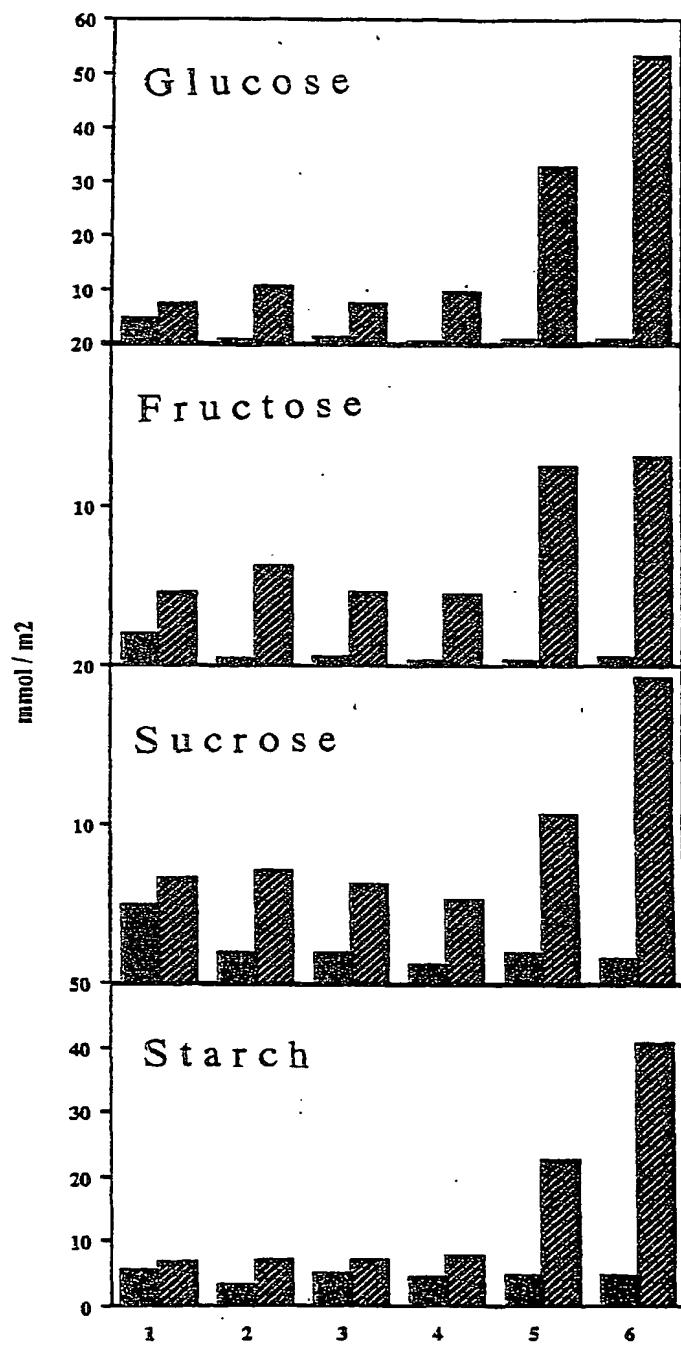


Fig. 4

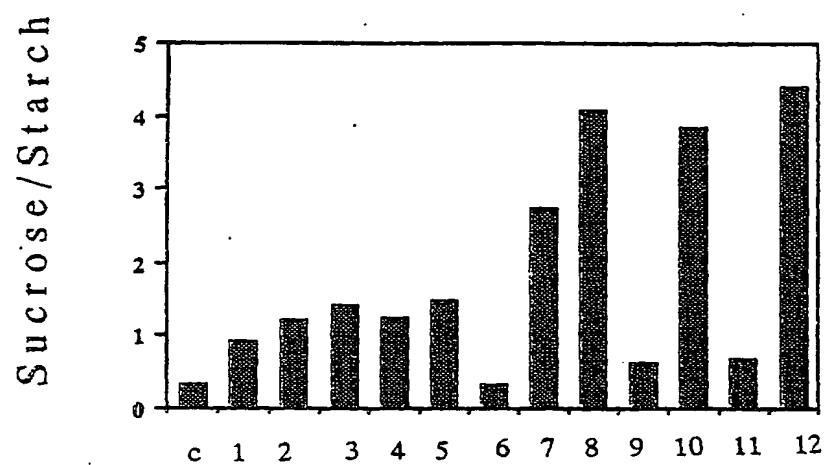


Fig. 5

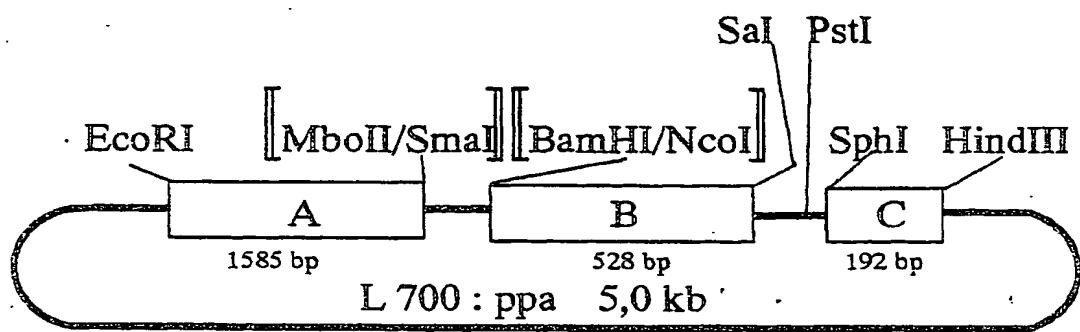


Fig. 6

(19)



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(11)

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(12)

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**(54) Plasmids for the production of transgenic plants that are modified in habit and yield**

(57) Plasmids that comprise a DNA sequence cod-  
ing for a protein that modifies the metabolism of soluble  
sugars, for example, an inorganic pyrophosphatase  
agent and plant cells comprising those plasmids, for the

production of transgenic plants that are modified in habit  
and yield are described, the plants being modified in  
habit by the transfer and the expression of genes that  
intervene in sugar metabolism or sugar distribution in-  
side a plant.

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| DOCUMENTS CONSIDERED TO BE RELEVANT  |  |                       |  |                              |   |                       |  |  |  |
|--|--|-----------------------|--|------------------------------|---|-----------------------|--|--|--|
| Category   | Citation of document with indication, where appropriate, of relevant passages  | Relevant to claim     | CLASSIFICATION OF THE APPLICATION (Int.Cl.7) |                              |   |                       |  |  |  |
| X  | <p>BRITTEN C J ET AL: "IDENTIFICATION AND PURIFICATION OF SUBSTRATE-BINDING SUBUNIT OF HIGHER PLANT PROTON-TRANSLOCATING INORGANIC PYROPHOSPHATASE"<br/>FEBS (FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES) LETTERS, vol. 256, no. 1-2, 1989, pages 200-206, XP002178878<br/>ISSN: 0014-5793<br/>* the whole document *</p> <p>---</p> <p>X DATABASE BIOSIS 'Online!<br/>BIOSCIENCES INFORMATION SERVICE,<br/>PHILADELPHIA, PA, US; 1988<br/>MOHABIR G ET AL: "EFFECT OF TEMPERATURE ON STARCH SYNTHESIS IN POTATO TUBER TISSUE AND IN AMYLOPLASTS"<br/>Database accession no. PREV198987075818<br/>XP002178880<br/>* abstract *<br/>&amp; PLANT PHYSIOLOGY (BETHESDA), vol. 88, no. 4, 1988, pages 1222-1228, ISSN: 0032-0889<br/>---</p> <p>A LAHTI R ET AL: "CLONING AND CHARACTERIZATION OF THE GENE ENCODING INORGANIC PYROPHOSPHATASE OF ESCHERICHIA-COLI K-12"<br/>JOURNAL OF BACTERIOLOGY, vol. 170, no. 12, 1988, pages 5901-5907, XP002178879<br/>ISSN: 0021-9193<br/>* the whole document *</p> <p>---</p> <p>---</p> | 9, 10                 | C12N15/55<br>C12N15/82<br>A01H5/00           |                              |   |                       |  |  |  |
|  |  |                       | TECHNICAL FIELDS SEARCHED (Int.Cl.7)         |                              |   |                       |  |  |  |
|  |  |                       | C12N   |                              |   |                       |  |  |  |
| <p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search<br/>THE HAGUE</td> <td>Date of completion of the search<br/>28 September 2001</td> <td>Examiner<br/>Maddox, A</td> </tr> <tr> <td colspan="3"> <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone<br/>Y : particularly relevant if combined with another document of the same category<br/>A : technological background<br/>O : non-written disclosure<br/>P : Intermediate document</p> <p>T : theory or principle underlying the invention<br/>E : earlier patent document, but published on, or after the filing date<br/>D : document cited in the application<br/>L : document cited for other reasons</p> <p>B : member of the same patent family, corresponding document</p> </td> </tr> </table> |  |                       |  | Place of search<br>THE HAGUE | Date of completion of the search<br>28 September 2001 | Examiner<br>Maddox, A | <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone<br/>Y : particularly relevant if combined with another document of the same category<br/>A : technological background<br/>O : non-written disclosure<br/>P : Intermediate document</p> <p>T : theory or principle underlying the invention<br/>E : earlier patent document, but published on, or after the filing date<br/>D : document cited in the application<br/>L : document cited for other reasons</p> <p>B : member of the same patent family, corresponding document</p> |  |  |
| Place of search<br>THE HAGUE   | Date of completion of the search<br>28 September 2001  | Examiner<br>Maddox, A |  |                              |   |                       |  |  |  |
| <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone<br/>Y : particularly relevant if combined with another document of the same category<br/>A : technological background<br/>O : non-written disclosure<br/>P : Intermediate document</p> <p>T : theory or principle underlying the invention<br/>E : earlier patent document, but published on, or after the filing date<br/>D : document cited in the application<br/>L : document cited for other reasons</p> <p>B : member of the same patent family, corresponding document</p>   |  |                       |  |                              |   |                       |  |  |  |



| DOCUMENTS CONSIDERED TO BE RELEVANT   |  |   |   |
|---|--|---|---|
| Category  | Citation of document with indication, where appropriate, of relevant passages  | Relevant to claim   | CLASSIFICATION OF THE APPLICATION (Int.Cl.) |
| A   | <p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1989</p> <p>QUICK P ET AL: "FLUORIDE LEADS TO AN INCREASE OF INORGANIC PYROPHOSPHATE AND AN INHIBITION OF PHOTOSYNTHETIC SUCROSE SYNTHESIS IN SPINACH LEAVES"</p> <p>Database accession no. PREV198987108500</p> <p>XP002178881</p> <p>* abstract *</p> <p>&amp; BIOCHIMICA ET BIOPHYSICA ACTA, vol. 973, no. 2, 1989, pages 263-271, ISSN: 0006-3002</p>                       | 1-12  |   |
| A   | <p>SCHAEWEN VON A ET AL: "EXPRESSION OF A YEAST-DERIVED INVERTASE IN THE CELL WALL OF TABACO AND ARABIDOPSIS PLANTS LEADS TO ACCUMULATION OF CARBOHYDRATE AND INHIBITION OF PHOTOSYNTHESIS AND STRONGLY INFLUENCES GROWTH AND PHENOTYPE OF TRANSGENIC TOBACCO PLANTS"</p> <p>EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 9, no. 10, 1 October 1990 (1990-10-01), pages 3033-3044, XP000572662</p> <p>ISSN: 0261-4189</p> <p>* the whole document *</p> |   | TECHNICAL FIELDS SEARCHED (Int.Cl.)         |
| A   | <p>WO 89 12386 A (CALGENE INC)</p> <p>28 December 1989 (1989-12-28)</p> <p>* the whole document *</p>  |   |   |
| A   | <p>WO 90 12876 A (DANSKE SPRITFABRIKKER)</p> <p>1 November 1990 (1990-11-01)</p> <p>* examples 24,25 *</p>   |   |   |
| <p>The present search report has been drawn up for all claims</p>   |  |   |   |
| <p>Place of search</p> <p>THE HAGUE</p>   |  | <p>Date of completion of the search</p> <p>28 September 2001</p>  | Examiner                                    |
| <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone</p> <p>Y : particularly relevant if combined with another document of the same category</p> <p>A : technological background</p> <p>O : non-written disclosure</p> <p>P : Intermediate document</p> |  | <p>T : theory or principle underlying the invention</p> <p>E : earlier patent document, but published on, or after the filing date</p> <p>D : document cited in the application</p> <p>L : document cited for other reasons</p> <p>&amp; : member of the same patent family, corresponding document</p> |   |

ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.

EP 01 10 6521

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

28-09-2001

| Patent document cited in search report |   | Publication date |    | Patent family member(s) | Publication date |
|--|---|------------------|----|-------------------------|------------------|
| WO 8912386                             | A | 28-12-1989       | AU | 3852089 A               | 12-01-1990       |
|  |   |                  | WO | 8912386 A1              | 28-12-1989       |
| WO 9012876                             | A | 01-11-1990       | AT | 104351 T                | 15-04-1994       |
|  |   |                  | AU | 638248 B2               | 24-06-1993       |
|  |   |                  | AU | 5531890 A               | 16-11-1990       |
|  |   |                  | CA | 2053230 A1              | 25-10-1990       |
|  |   |                  | DD | 298949 A5               | 19-03-1992       |
|  |   |                  | DE | 69008172 D1             | 19-05-1994       |
|  |   |                  | DE | 69008172 T2             | 20-10-1994       |
|  |   |                  | WO | 9012876 A1              | 01-11-1990       |
|  |   |                  | DK | 470145 T3               | 08-08-1994       |
|  |   |                  | EP | 0470145 A1              | 12-02-1992       |
|  |   |                  | ES | 2053189 T3              | 16-07-1994       |
|  |   |                  | IE | 63067 B                 | 22-03-1995       |
|  |   |                  | LV | 10727 A                 | 20-06-1995       |
|  |   |                  | LV | 10727 B                 | 20-02-1996       |
|  |   |                  | US | 5498832 A               | 12-03-1996       |
|  |   |                  | US | 5789657 A               | 04-08-1998       |
|  |   |                  | US | 6147277 A               | 14-11-2000       |